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Methotrexate modulates folate phenotype and inflammatory profile in EA.hy 926 cells

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Abstract

EA.hy 926 cells grown under low folate conditions adopt a “pro-atherosclerotic” morphology and biochemical phenotype. Pharmacologically relevant doses of the antifolate drug methotrexate (MTX) were applied to EA.hy 926 cells maintained in normal (Hi) and low (Lo) folate culture media. Under both folate conditions, MTX caused inhibition of cell proliferation without significantly compromising metabolic activity. MTX treated Hi cells were depleted of folate derivatives, which were present in altered proportions relative to untreated cells. Transcript profiling using microarrays indicated that MTX treatment modified the transcriptome in similar ways for both Hi and Lo cells. Many inflammation-related genes, most prominently those encoding C3 and IL-8, were up-regulated, whereas many genes involved in cell division were down-regulated. The results for C3 and IL-8 were confirmed by quantitative RT-PCR and ELISA. MTX appears to modify the inflammatory potential of EA.hy 926 cells such that its therapeutic properties may, at least under some conditions, be accompanied by the induction of a subset of gene products that promote and/or maintain comorbid pathologies.

Keywords

Inflammation; Folate; Methotrexate; Immune modulation; Gene expression

1. Introduction

Hyperhomocysteinemia, in which circulating concentrations of the intermediate amino acid homocysteine (Hcy) are elevated, has been associated with a wide range of human

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Disclosures

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pathologies including atherothrombotic diseases (Refsum et al., 1998), Alzheimer's disease (Mattson and Shea, 2003), some cancers (Weinstein et al., 2001), and the birth defect spina bifida (Mitchell et al., 2004). It is generally underpinned by low folate status (Jacques et al., 1996; Harmon et al., 1996) and the relative concentrations of intracellular folate derivatives may be altered (Mitchell et al., 2009). The folate/Hcy metabolic pathway is the means whereby one carbon units are channeled into important biological processes including methylation, glutathione production, and nucleic acid synthesis (Lucock, 2006) (Figure 1). The key folate derivative, 5-methyltetrahydrofolate (5-MTHF), provides the methyl group for the remethylation of Hcy to methionine. The latter is subsequently converted to S-adenosylmethionine (SAM), the methyl donor for many methyltransferase reactions on substrates such as DNA, proteins, and lipids. The loss of the methyl group from 5-MTHF generates tetrahydrofolate (THF) which is in turn converted to 5,10-methyleneTHF. This derivative can be reduced by 5,10-methylenetetrahydrofolate reductase (MTHFR) to regenerate 5-MTHF or used to initiate a series of reactions to generate thymidylate and purines. Historically, elevated Hcy was considered to be the pathogenic component in the conditions with which hyperhomocysteinemia has been associated because of its direct toxic effects on redox thiol status and ER stress response (Koch et al., 1998). However, alternative causative mechanisms implicating low folate concentrations and their negative impact on processes such as nucleic acid synthesis and methylation have been suggested (Lucock, 2000).

Many of the above pathologic conditions have inflammatory aspects and involve damage to, or dysfunction of, the vasculature and its constituent cell types, in particular endothelial cells. Inappropriate or sustained activation of immunologically active endothelial cell products might contribute to ongoing pathology at the local and possibly also systemic level. In recent studies EA.hy 926 cells, which are derived from the fusion of primary endothelial cells and an epithelial tumor cell line but have an endothelial-like morphology and synthetic profile, were grown under low folate conditions and adopted a "pro-atherosclerotic" phenotype compared to cells grown under high folate conditions, without displaying any significant differences in intracellular or extracellular Hcy levels (Brown et al., 2006). This phenotype was characterized by elongated cell morphology with prominent networks of stress fibers and impaired barrier function. A significantly increased synthesis and export of monocyte chemoattractant protein 1 (MCP-1) was observed in the cells grown under low folate conditions (Brown et al., 2006). MCP-1, a potent chemokine that is synthesized by vascular smooth muscle cells in response to oxidized lipid, facilitates the transmigration of monocytes from the circulation across the endothelium and is a key contributor to the early stages of atheroma formation (Schwartz et al., 1991). In cultured human monocytes Hcy has been shown to induce the secretion of IL-8, a neutrophil chemoattractant, as well as MCP-1 (Zeng et al., 2003). The above *in vitro* observations have been corroborated *in vivo* in a study of young healthy adults in whom serum MCP-1 levels were inversely associated with serum and red blood cell folate concentrations, and positively associated with circulating Hcy concentrations (Hammons et al., 2009). Taken together, these findings have reinforced speculation that "folate stress," which is indicative of poor nutritional status, might augment aspects of baseline inflammatory preparedness to facilitate more vigorous initial responses to infectious challenges in individuals weakened by malnutrition (Lu et al., 2009). Such an

adaptive response may have evolved if the consequent survival advantage offsets the negative effects of enhanced sub-clinical inflammatory processes.

The central role of folate in nucleotide synthesis has been exploited pharmacologically via the development of potent antifolate drugs for the treatment of neoplastic and auto-immune conditions. One of the most widely used antifolate drugs is methotrexate (MTX), which inhibits the key enzymes dihydrofolate reductase (DHFR), thymidylate synthase (TYMS), glycinamide ribonucleotide transformylase (GART), and aminoimidazolecarboxamide ribonucleotide transformylase (AICART) (Kremer, 2004) (Figure 1). High dose MTX is a component of diverse therapeutic regimens for several cancers including acute lymphoblastic leukemia (Jonsson and Kamen, 1991), while lower doses are used to treat inflammatory diseases such as rheumatoid arthritis (RA) (Williams et al., 1985). In the latter condition, MTX tends to be well tolerated with relatively minor side effects and there is clear therapeutic benefit in reducing the inflammatory aspects of the disease that contribute to joint damage (Coury and Weinblatt, 2010). However, RA patients have significant cardiovascular comorbidity (Nurmohamed, 2009) and there is controversy as to whether MTX exacerbates or ameliorates this serious source of mortality. An early study on the use of MTX in the treatment of rheumatoid arthritis patients with existing CVD indicated that mortality was increased (Landewe et al., 2000). Conversely, several more recent studies have suggested that MTX use is associated with a decrease in the incidence of CVD events and mortality (Choi et al., 2002; van Halm et al., 2006; Naranjo et al., 2008), although it remains unclear whether such a decrease would reflect a full or only partial amelioration of inflammation-attributable CVD.

The possibility that low folate status, due to nutritional variables or the use of antifolate drugs, contributes to human disease by inducing a subset of potentially pathogenic inflammation-associated molecules, including MCP-1, is of considerable public health interest. The characterization of changes to the inflammatory profile that might be induced by drugs such as MTX would serve as the foundation for future studies to define the precise relationship between dysregulation of folate metabolism and inflammation. This study was designed to investigate the effect of pharmacologically relevant doses of MTX on the absolute and relative concentrations of key folate derivatives and gene expression in the Ea.hy 926 cell line. The potential implications of observations concerning the up-regulation of key inflammatory proteins are discussed.

2. Materials and Methods

2.1 Cell Culture

EA.hy 926 cells (Edgell et al., 1983) are a fusion product between human umbilical vascular endothelial cells (HUVECs) and the epithelial cell line A549 derived from a human lung carcinoma. EA.hy 926 cells were adapted to growth under low folate conditions (Lo cells) in Medium 199 (Gibco, Invitrogen, Carlsbad, CA), which contains 23 nM of folic acid, supplemented with 10% FCS, non-essential amino acids, gentamycin, penicillin G, and fungizone. Parallel cultures of EA.hy 926 cells were grown under standard folate concentrations for that cell line (Hi cells), in Medium 199 with folic acid increased to 9 μ M and supplemented as above (Brown et al., 2006).

2.2 BrdU Cell Proliferation Assays

Hi and Lo cells were seeded into 96-well plates in their respective media at a density that would yield 50% confluence after overnight incubation. Triplicate cultures were then maintained in fresh media containing 0, 0.1, 0.25, or 0.5 μM MTX (Sigma-Aldrich, St. Louis, MO) for 24 and 48 h, after which media were removed and adherent cells were fixed and stained using the Cell Proliferation ELISA, BrdU Colorimetric kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. Colorimetric analyses were performed with an ELISA plate reader (Dynex Technologies, Chantilly, VA).

2.3 Cell Viability Assays

Hi and Lo cells, grown to confluence in 6-well plates, were maintained for 24 h in fresh medium prior to the addition of 0, 0.1, 0.25, or 0.5 μM MTX. After a further 48 h the numbers of live cells remaining were determined in duplicate with an electronic cell counter (Scepter, Millipore, Bedford, MA). The numbers of live and dead cells in each treatment group were also determined by counting using a hemacytometer and a dye exclusion assay using 0.4% w/v trypan blue solution (Mediatech, Herndon, VA).

2.4 Alamar Blue Assays

Fresh medium was added to confluent Hi and Lo cell cultures grown in 96-well plates, and treated 24 h later with 0, 0.1, 0.25, or 0.5 μM MTX. After 2, 8, 16, 24, and 48 h metabolic activity was measured in biological triplicates by Alamar Blue Assay (Trek Diagnostic Systems, West Lake, OH) according to the manufacturer's directions.

2.5 Biochemical Phenotyping

Confluent Hi and Lo cells were maintained for 24 h in fresh medium prior to treatment with 0.5 μM MTX for 48 h. Intracellular folate derivatives, i.e. 5-MTHF, THF, 5,10-methenyltetrahydrofolate (5,10-MTHF), and unmetabolized folic acid (FA), were measured in biological triplicates by stable isotope dilution liquid chromatography, multiple reaction monitoring, mass spectrometry (LC/MRM/MS) as described previously (Huang et al., 2008).

2.6 Affymetrix Microarrays

RNA was isolated from biological triplicates with the RNeasy kit (Qiagen Inc., Valencia, CA) and reverse transcribed to cDNA using the Affymetrix WT Expression kit (Ambion, Austin, TX). Subsequent experimental procedures and statistical analyses of microarray signals were performed by the University of Pennsylvania Microarray and Bioinformatics core facilities respectively as follows. The purity and size distribution of cDNA products were assessed using the Agilent Bioanalyzer and RNA6000 Nano LabChips (Agilent, Palo Alto, CA), and quantitation was performed using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE), prior to their hybridization to Affymetrix Human Gene 1.0 ST microarrays. Washing and staining of the microarrays was done on an Affymetrix 450 series fluidics machine. Scanning of each microarray was performed with an Affymetrix Gene Chip Scanner 3000 to produce Cel files that were imported into Partek Genomics Suite v6.5 (Partek Inc., St Louis, MO) where robust multi-array analysis was applied. A fold change of

>2 and false discovery rate of 5%, corresponding to a corrected P-value of <0.05 were chosen as the cutoffs. The former criterion was relaxed in order to identify the top 200 significantly up-regulated and down-regulated transcripts. Inflammation-related and other classes of genes were identified as such based on the GO annotation provided by Affymetrix. The microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO, <http://www.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE32008.

2.7 Quantitative Real Time PCR (qRT-PCR)

RNA was prepared from biological triplicates of control and MTX treated Hi and Lo cells using the RNeasy kit (Qiagen Inc.). Reverse transcription was carried out with MMLV reverse transcriptase (Promega, Madison, WI) as described previously (Brown et al., 2006). To determine the amount of target mRNA relative to that transcribed from the housekeeping gene *GAPDH*, qRT-PCR was carried out in 20 µl reactions containing 1 µl cDNA, 1 µl Taqman Gene Expression Assay (Applied Biosystems, Foster City, CA) in Taqman Universal master mix (Applied Biosystems). The Applied Biosystems assay ID numbers were Hs01100879_m1 for C3, Hs00174103_m1 for IL-8 (encoded by *CXCL8*), and Hs00758822_s1 for DHFR. *GAPDH* and MCP-1 (encoded by *CCL2*) qRT-PCR assays were performed as previously described (Brown et al., 2006). All samples were assayed in duplicate.

2.8 ELISA Assays

Confluent Hi and Lo cells were maintained for 24 h in fresh medium prior to treatment with 0.5µM MTX. After a further 48 h MCP-1, IL-8, and C3 concentrations in medium from MTX treated and untreated control cells were measured in biological triplicates using MCP-1 (PeproTech, Inc, Rocky Hill, NJ), IL-8 (BD Biosciences, San Diego, CA), and C3 (Innovative Research, Novi, MI) ELISA kits, with adjustment for protein content in the corresponding cell fraction. All individual samples were assayed in duplicate.

2.9 Statistical Methods

Differences between means were compared by two-tailed Student's t-test. Statistical analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC).

3. Results

3.1 MTX Dose Finding

Peak concentrations of plasma MTX in patients undergoing treatment for RA have been reported to fall within a range of 0.37–1.36 µM (Lebbe et al., 1994). This guided our selection of test MTX doses (i.e. 0.1, 0.25, and 0.5 µM) that were applied to Ea.hy 926 cells to identify a concentration that was pharmacologically relevant but did not cause excessive cell death or compromise overall metabolic activity. Several methods were used as detailed below.

3.2 The Effect of MTX on EA.hy 926 Cell Proliferation

BrdU cell proliferation assays were performed on Hi and Lo cells following 24 and 48 h of treatment with all test doses of MTX. A dose dependent inhibition of proliferation in MTX treated Hi cells was observed relative to control untreated cells at both time points (Figure 2A), whereas MTX significantly inhibited proliferation at all concentrations tested in Lo cells at 48 h ($p<0.05$) (Figure 2B). Comparable levels of inhibition (approximately 84%) were observed for both Hi and Lo cell cultures using the 0.5 μM dose of MTX.

3.3 The Effect of MTX on EA.hy 926 Cell Viability

The proportion of Hi and Lo cells that remained viable after treatment with 0.5 μM MTX for 48 h was determined using two independent methods. By Trypan Blue exclusion assay, the percentages of live treated and untreated Hi cells were 96% and 99% respectively, and the percentages of live treated and untreated Lo cells were 67% and 96% respectively (data not shown). Direct counting using an electronic cell counter indicated that MTX-treated Hi cells had 93% as many live cells as controls ($P=0.26$); for MTX-treated Lo cells this figure was 62% ($P=0.001$) (data not shown). Thus, by both methods 0.5 μM MTX had only a minimal impact on Hi cell viability and a moderate impact on Lo cell viability.

3.4 The Effect of MTX on EA.hy 926 Cell Metabolic Activity

Alamar Blue assays were performed on confluent Hi and Lo cell cultures following treatment with all test concentrations of MTX (i.e. 0.1, 0.25, 0.5 μM) for 2, 8, 16, 24, and 48 h in order to estimate reductions in basic metabolic activity over time. None of the MTX concentrations had a substantial impact on metabolic activity in either series (Figure 2C and 2D). Hi and Lo cells treated with the highest dose of MTX (0.5 μM) for 48 h retained more than 79% ($P=0.005$) and 82% ($P=0.029$) of the untreated control Alamar Blue readouts respectively, indicating that metabolism remained largely intact in MTX treated cells.

Taken together the above results suggested that MTX at a concentration of 0.5 μM would be an appropriate, pharmacologically relevant dose at which to explore the impact of antifolate drug treatment on folate phenotype and gene expression profile.

3.5 Modulation of Folate Phenotype in EA.hy 926 Cells by MTX

Exposure of confluent Hi and Lo EA.hy 926 cell cultures to 0.5 μM MTX for 48 h resulted in qualitative and quantitative changes to folate phenotype in both. In Hi cells there was an almost 20 fold increase in unmetabolized FA from a mean of 32.9 ng per mg protein in untreated cells to 630.4 ng per mg protein following MTX treatment ($P=0.003$, Figure 3A). This indicated that Hi cells were able to take up FA from the medium in the presence of MTX, and that the drug efficiently inhibited DHFR activity to prevent the step-wise conversion of FA to DHF and THF and its subsequent entry into the cellular pool of natural folates. The accumulation of FA in MTX treated Hi cells was accompanied by a quantitative reduction in total intracellular folate (i.e. the sum of the three derivatives 5-MTHF, THF, and 5,10-MTHF) from 2099.1 ng per mg protein in untreated cells to 144.2 ng per mg protein in MTX treated cells ($P=0.003$). This represented a reduction of total folates in Hi cells to only 7% of levels observed in untreated control cells. Although the concentrations of each of the individual folate analytes also fell (i.e. 5-MTHF from 901.8 to 8.9 ng per mg

protein, $P<0.001$; THF from 506.3 to 45.5 ng per mg protein, $P=0.001$; and 5,10-MTHF from 690.0 to 89.8 ng per mg protein, $P=0.008$) the extent to which they did so differed. MTX treated Hi cells had 5-MTHF, THF, and 5,10-MTHF concentrations that were respectively 1%, 9%, and 13% those observed in untreated cells. Thus the individual folates represented very different proportions of total folate in untreated Hi cells compared to MTX treated Hi cells (43% to 6% for 5-MTHF, 24% to 32% for THF, and 33% to 62% for 5,10-MTHF).

FA levels were estimated to be 12.4 ng per mg protein in untreated Lo cells compared to 22.6 ng per mg protein in MTX treated Lo cells (Figure 3B). Although this change was not statistically significant the trend towards higher concentrations in the latter suggests that Lo cells were able to take up FA in the presence of MTX but were unable to process it due to inhibition of DHFR. Total folate levels did not change significantly (102.1 and 107.5 ng per mg protein in untreated and MTX treated Lo cells, respectively). Concentrations of the individual folate derivatives also did not change substantially (5-MTHF levels were 16.8 and 15.8 ng per mg protein, THF levels were 12.4 and 15.8 ng per mg protein, and 5,10-MTHF levels were 72.9 and 75.8 ng per mg protein in untreated and MTX treated Lo cells, respectively).

3.6 MTX-Attributable Changes in Gene Expression

The impact of MTX on gene expression was assessed in both Hi and Lo cells using Affymetrix Human Gene 1.0 ST microarrays. An arbitrary threshold of at least 2 fold change in microarray signal relative to that of untreated controls and an adjusted P value <0.05 was used to identify transcripts of interest.

There were 46 genes in MTX treated Hi cells and 48 genes in MTX treated Lo cells that were up-regulated and met the above criteria (Supplementary Table 1); 36 were in common, of which 7 have inflammation-related functions (complement component C3 [C3], interleukin 1 alpha, TNF receptor-associated factor 1, CEBP beta, TNF receptor superfamily member 9, interleukin 11, leukemia inhibitory factor, interleukin 32). When the fold change selection criterion was relaxed to identify the top 200 up-regulated transcripts, there were 22 and 18 inflammation-related genes which appeared in the ranking for MTX treated Hi and Lo cells, respectively (i.e. approximately 10% of the total in each case; Supplementary Table 1).

Two inflammatory genes, C3 and IL-8, ranked towards the top of the lists of overall up-regulated transcripts in both MTX treated Hi and Lo cells. C3 ranked first for Hi cells (up-regulated 6.09 fold, $P=0.005$) and third for Lo cells (up-regulated 4.26 fold, $P=0.013$) (Table 1). IL-8 ranked sixth for Hi cells (up-regulated 4.06 fold, $P=0.036$) and eleventh for Lo cells (up-regulated by 3.00 fold, $P=0.095$). C3 and IL-8 are noteworthy for their important roles in complement activation and chemokine signaling respectively, and each is implicated as a key driver of some inflammation-associated pathologies.

The increased levels of both C3 and IL-8 mRNAs following MTX treatment was confirmed by qRT-PCR (Figure 4A and 4B, respectively). C3 mRNA levels were up-regulated in MTX treated Hi ($P=0.018$) and Lo ($P=0.003$) cells; IL-8 mRNA levels were up-regulated in MTX

treated Hi (P=0.041) and Lo (P=0.015) cells. In addition, each of these mRNA species was significantly more abundant in Lo untreated cells than in Hi untreated cells (P=0.034 for C3; P=0.010 for IL-8).

Another inflammatory gene, MCP-1, that was previously shown to be regulated by folate status (Brown et al., 2006), was not significantly different in the microarray analysis of MTX treated Hi or Lo cells compared to their respective controls (data not shown).

Following MTX treatment, expression was reduced by at least 50% for 83 genes in Hi cells and 85 genes in Lo cells (Supplementary Table 2); of these 69 were in common. Among the most prominently down-regulated transcripts several were cell cycle related products. In addition mRNA encoding DHFR, the primary enzyme inhibited by MTX, was down-regulated by more than 2 fold in both Hi (P=0.003) and Lo cells (P=0.002) after treatment with MTX. The MTX associated down-regulation of DHFR mRNA levels was confirmed by qRT-PCR (1.9 fold lower in Hi cells, P=0.010; 3.6 fold in Lo cells, P=0.009, data not shown).

The readout from the gene expression analyses reported here has been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO, <http://www.nlm.nih.gov/geo/>) and is accessible through GEO Series accession number GSE32008.

3.7 MTX-Attributable Changes in C3, IL-8, and MCP-1 Protein Levels

C3, IL-8, and MCP-1 protein concentrations were measured in media from untreated Hi and Lo cells and in Hi and Lo cells treated with 0.5 μ M MTX for 48 h. Secreted C3 levels were 3.0 fold higher in MTX treated Hi cell medium (P=0.032) and 1.3 fold higher in MTX treated Lo cell medium (P=0.018) relative to their respective control media (Figure 5A). Secreted IL-8 levels were up-regulated 12.5 fold in MTX treated Hi cell medium (P=0.016) and 1.7 fold in MTX treated Lo cell medium (P=0.008) (Figure 5B). Secreted MCP-1 levels were not significantly different in MTX treated Hi or Lo cells compared to their respective untreated controls (Figure 5C). Relative to untreated Hi cell medium, untreated Lo cell medium had significantly more secreted C3 (5.1 fold more, P=0.001), IL-8 (6.8 fold more, P=0.007), and MCP-1 (1.7 fold more, P=0.033).

4. Discussion

MTX is widely used as a therapeutic agent for the treatment of some common cancers, including breast and lung cancer, and some auto-immune inflammatory diseases, including rheumatoid arthritis. At high doses the effect of MTX is anti-proliferative; at low doses it ameliorates the aberrant immune activation that is a feature of auto-immune diseases. Patients who have such conditions often have comorbidities such as cardiovascular disease, even in the absence of MTX therapy (Sattar et al., 2003). It remains unclear whether MTX therapy reduces or increases the risk of comorbidity, and if so whether it acts via direct effects on inflammation or through indirect mechanisms.

Antifolate drugs like MTX restrict the amount of intracellular folate and modify the distribution of folate derivatives that individually support important functions such as

methylation and nucleic acid synthesis. Like low folate status itself, such pharmacologically mandated “folate stress” has the potential to cause pathogenic changes to cell phenotype. We have previously used the endothelial-like cell line EA.hy 926 to investigate the consequences of low folate culture conditions on cell phenotype. In addition to morphologic changes, cells grown under low folate conditions make increased amounts of MCP-1, a potent pro-inflammatory chemokine involved in early atherogenesis, compared to cells grown under folate replete conditions (Brown et al., 2006). Suboptimal folate culture conditions (i.e. folate stress) may therefore mobilize aspects of immune/inflammatory responses that have pathogenic potential. The above *in vitro* observations have been corroborated *in vivo* in a study of healthy young adults in whom serum MCP-1 levels were inversely associated with serum and RBC folate concentrations, and positively associated with circulating Hcy concentrations (Hammons et al., 2009). This up-regulation of MCP-1 is underpinned, at least in part, by the stress-activated p38 MAPK pathway (Lu et al., 2009). The above observations have led us to speculate that low folate status, which is indicative of poor nutrition and susceptibility to infectious disease, might prime some components of immune and inflammatory responses to be present at higher baseline concentrations to permit more vigorous initial responses to infectious challenges. We set out to determine whether MTX treatment can induce biosynthetic changes that are analogous to those caused by “nutritionally-driven” folate stress (i.e. are “MTX-driven” quantitative and qualitative changes in folate metabolism associated with mobilization of aspects of the immune/inflammatory response?). The EA.hy 926 cell line was used to test this question *in vitro* employing therapeutically relevant MTX doses in the context of low and high folate culture conditions. The qualitative and quantitative impact of MTX on intracellular folates and the accompanying changes in gene expression were assessed.

Our data indicated that relatively low MTX concentrations (similar to the peak plasma concentrations observed after low dose therapy (Lebbe et al., 1994) in patients with autoimmune diseases such as rheumatoid arthritis) can induce biologically significant effects in Ea.hy 926 cells. In cells grown under both high and low folate conditions the inhibition of DHFR led to an intracellular accumulation of unmetabolized folic acid and a consequent reduction in the amount of total intracellular folate. In cells grown under folate replete conditions this overall quantitative effect represented the sum of differential reductions in 5-MTHF, THF, and 5,10-MTHF concentrations such that the relative proportions of these derivatives were altered. The most striking change was for 5-MTHF, the most abundant form in untreated cells, which became the least abundant after MTX treatment.

The nature of the quantitative and qualitative changes elicited by MTX treatment might be expected to modify the efficiencies of folate-dependent functions such as synthesis of nucleic acids, production of glutathione, and methylation of substrates including DNA. The last of these could underpin altered gene expression that would be amenable to experimental observation. Following the application of a pharmacologically relevant dose of MTX, sufficient to inhibit cell division but not to cause undue cytotoxicity, such changes in gene expression were observed in cells maintained in both high and low folate culture media. As might be expected from its anti-proliferative effect, MTX treatment was associated with the down-regulation of a wide range of transcripts involved in cell division and cell cycle

progression. In contrast, immune/inflammation-related transcripts were prominent among up-regulated transcripts both numerically and in terms of fold-increase over control transcript levels, suggesting that a subset of products involved in immune and inflammatory processes (in particular those involved in signaling) are concurrently induced by MTX-driven folate stress. The highest ranked of these transcripts were C3 and IL-8. C3 is the lynchpin component of complement and has multiple functions, including the promotion of local inflammation when activated; increased levels of C3 are found in synovial fluids of patients with rheumatoid arthritis (Moxley and Ruddy, 1985). IL-8 is a chemokine that has potent chemotactic properties targeted at many different immune cells; its pro-inflammatory actions lead to local increases in mediators of oxidant stress. In control (i.e. untreated) cells grown under low folate conditions C3 and IL-8 were more highly expressed and, like MCP-1, had increased synthesis and export of protein compared to control cells grown under high folate conditions. This finding reinforces our previously published hypothesis that folate stress *per se* is associated with activation of certain aspects of immune function that are protective in the short term but may be deleterious when chronically maintained. Interestingly, the increased synthesis of MCP-1 under low folate conditions, but not in response to MTX, contrasts with the up-regulation of C3 and IL-8 following either challenge, and suggests that subtly different control pathways may be triggered or modified by nutritionally- and pharmacologically-driven “folate stress”.

The up-regulation of inflammatory genes, including C3 and IL-8 following MTX treatment is an intuitively paradoxical finding given the long established use of this and other antifolate drugs to treat diseases with a predominantly inflammatory nature. However, there are published reports of several inflammatory comorbidities being associated with MTX use. For example, pneumonitis, a severe inflammatory lung condition that is an occasional side effect of MTX therapy has been shown to be related to an elevation of IL-8 (Yoshida et al., 1999), possibly involving direct MTX-driven up-regulation of IL-8 by bronchial and alveolar epithelial cells (Yamauchi et al., 2004). This exemplifies the potential for MTX to initiate and maintain comorbid pathologies through immune/inflammatory mechanisms. Nevertheless, the positive therapeutic benefits of MTX and other antifolate drugs are undisputed and their continued use is beyond question. Hence, future studies of this class of drugs might benefit from the incorporation of surveillance of key inflammation-related mediators and biomarkers that may, in some individuals, contribute to comorbidities. Such studies might eventually lead to the identification of patients at risk of serious drug-related side effects, facilitating the substitution of other clinically relevant medications and/or the development of dosing and monitoring strategies to mitigate the pathogenic aspects of antifolate therapy without compromising efficacy, thereby supporting a personalized medicine approach to a spectrum of common human diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

5-MTHF	5-methyltetrahydrofolate
5,10-MTHF	5,10-methenyltetrahydrofolate
CVD	cardiovascular disease
FA	folic acid
Hcy	homocysteine
MTX	methotrexate
THF	tetrahydrofolate

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Folate/Homocysteine pathway. 5-MTHF, 5-methyltetrahydrofolate; 5,10-MTHF, 5,10-methenyltetrahydrofolate; DHF, dihydrofolate; DHFR, dihydrofolate reductase; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; Hcy, homocysteine; MTX, methotrexate; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate; TYMS, thymidylate synthase.

2A

2B

2C

2D

Figure 2.

The effects of MTX dose on the proliferation and metabolic activity of EA.hy 926 cells maintained in Hi and Lo folate media. *A* and *B*, Newly plated EA.hy 926 Hi (*A*) and Lo (*B*) cells were incubated overnight and plating medium was replaced with Hi or Lo medium, respectively, containing 0, 0.1, 0.25, or 0.5 μ M MTX. After 24 and 48 h the medium was removed and adherent cells were fixed and stained according to the manufacturer's instructions for BrdU quantitation. Each bar represents the mean $\pm$ S.D. of three samples. This experiment is representative of a total of three experiments with similar results. *C* and *D*, Confluent Hi (*C*) and Lo (*D*) cells were incubated with 0, 0.1, 0.25, or 0.5 μ M MTX. Cells were incubated with alamar blue for the 2 h immediately prior to the reading of absorbance at the following time points: 2, 8, 16, 24, and 48 h of exposure to MTX. Absorbance was measured at 570nm with a correction at 630nm and calculated as the percentage of control. Each bar represents the mean $\pm$ S.D. of three samples. This experiment is representative of a total of three experiments with similar results. *P values <0.05 compared with control at the same time point.

Figure 3.

The effect of MTX on FA and the folate derivatives: 5-MTHF, THF, and 5,10-MTHF.

Folate derivatives were measured by LC/MRM/MS in lysates from confluent Hi (A) and Lo (B) cells following treatment with 0.5 μ M MTX for 48 h. Each bar represents the mean $\pm$ S.D. of three samples. This experiment is representative of a total of three experiments with similar results except that in the other two experiments MTX treated Lo cells had slightly decreased levels of folate derivatives relative to control cells. *P values <0.05 compared with control.

Figure 4.

C3 and IL-8 mRNA levels in Hi and Lo cells treated with 0.5 μ M MTX for 48 h. C3 and IL-8 mRNA levels were assessed by qRT-PCR using the mRNA levels of the endogenous housekeeping gene *GAPDH* for normalization. Each bar represents mean $\pm$ S.D. target mRNA expression levels normalized to *GAPDH* mRNA levels of three samples. This experiment is representative of a total of three experiments with similar results; C3 mRNA (A), IL-8 mRNA (B). *P values <0.05 compared with respective control. #P values <0.05 for untreated Hi cells compared to untreated Lo cells.

Figure 5.

Secreted C3, IL-8, and MCP-1 protein levels in Hi and Lo cell media. Hi and Lo cells were treated with 0.5 μ M MTX for 48 h. C3 (A), IL-8 (B) and MCP-1 (C) were measured by ELISA. Each bar represents the mean $\pm$ S.D. of three samples. These data are representative of a total of three experiments with similar results. *P values <0.05 compared with respective control. #P values <0.05 for untreated Hi cells compared to untreated Lo cells.